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Cytoskeletal regulation of platelet formation: Coordination of F-actin and microtubules

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Signalling Network Facts

- Blood platelets are released into the circulation from their progenitor cell, the megakaryocyte which resides in the bone marrow.
- The process of platelet production involves maturation of megakaryocytes via endomitosis and the release of platelets from proplatelet extensions. Disruption of these processes can give rise to thrombocytopenia and/ or platelet function disorders.
- The actin and microtubule cytoskeletons are essential for proper maturation and proplatelet formation.
- Recent evidence has highlighted new roles for several proteins (e.g. WASp, Profilin, Pak2) in coordinating actin and microtubules to regulate platelet production.

Abstract

Platelets are small, anucleate blood cells which play an important role in haemostasis. Thrombocytopenia is a condition where the platelet count falls below 150×10^9 /litre and patients suffering from severe forms of this condition can experience life-threatening bleeds requiring platelet transfusions. Platelets are produced from large progenitor cells called megakaryocytes which are found in the bone marrow. The process of megakaryocyte maturation and the formation of proplatelets are essential steps in the production of mature platelets and both depend heavily on the actin and microtubule cytoskeletons. Understanding these processes is important for the development of *in vitro* platelet production which will help to treat thrombocytopenia as well as produce model systems for studying platelet-associated disorders. This review will highlight some of the recent advances in our understanding of the role of the cytoskeleton in platelet production, especially the key molecules and signalling pathways that regulate actin and microtubule crosstalk.

Key Words

Megakaryocyte, Platelets, Proplatelet formation, Actin, Microtubules

1. Introduction

Platelets are small, anucleate, circulating blood cells which play a critical role in the process of haemostasis (the prevention of blood loss following injury). The activation of platelets in response to vascular injury, along with vasoconstriction and the coagulation cascade, ensure that blood loss is restricted and promotes repair of damaged vessel walls. The normal platelet count of blood ranges between $150 - 400 \times 10^9$ /litre and human platelets have a lifespan of 8–10 days (Giles, 1981; Leeksa and Cohen, 1955). The average healthy adult produces 10^{11} platelets/day to maintain this count. Thrombocytopenia is a condition where the platelet count falls below the 150×10^9 /litre threshold. This can be caused by a variety of factors ranging from genetic causes to drug-induced

thrombocytopenia. Patients suffering from thrombocytopenia experience a range of symptoms and, depending on the severity, may require platelet transfusions to counteract the risk of severe bleeding (Reviewed by Gauer and Braun, 2012).

Platelets are produced from their progenitor cell, the megakaryocyte (MK), in the bone marrow in response to the hormone thrombopoietin (TPO) (Reviewed by Thon and Italiano 2012; Machlus and Italiano 2013). In response to TPO, haematopoietic stem cells differentiate into MKs by differential regulation/expression of transcription factors (including GATA1, FOG1, RUNX1, FLI1 and NF-E2). This maturation is characterised by an increase in size of the MK ($50\mu\text{m} \geq 100\mu\text{m}$) and DNA ploidy level of the cell due to endomitosis (DNA replication without cell division). This increase in size allows the MK to accumulate RNA, protein, internal membrane pools and organelles for subsequent packaging into platelets.

The production of platelets from mature MKs occurs via the extension of proplatelets (PPs) into the bone marrow sinusoidal blood vessels and release of (pro)platelets into the blood. It is known that inhibition of cytoskeletal dynamics has a detrimental effect on platelet production (Tablin et al. 1990; Italiano et al. 1999). This review will highlight some recent developments in our understanding of the proteins and signalling pathways that regulate the cytoskeleton during MK maturation, proplatelet formation (PPF) and platelet release.

2. Functions

The formation of platelets from mature MKs can be separated into several stages (Figure 1) which are summarised below and highlight the key functions of the actin and microtubule (MT) cytoskeletons. In addition, Table 1 summarises the roles of the various cytoskeletal proteins in platelet production.

2.1 MK maturation

During maturation, MK progenitors, which reside in the bone marrow osteoblastic niche, increase in size and migrate to the vascular bone marrow niche. Migration of MKs to the perivascular space is driven by a gradient of the chemokine stromal cell-derived factor 1 (SDF-1) (Avecilla et al. 2004) and is dependent on the actin cytoskeleton with a specific role for non-muscle myosin IIA (Pecci et al. 2011). Furthermore, precise regulation of the actin cytoskeleton is required to allow endomitosis which is responsible for the increase in MK size and ploidy from 2N up to 128N.

2.2 Extension of proplatelets into the blood stream

Once in the vascular niche mature MKs produce protrusions called proplatelets (PPs) which extend across the sinusoidal blood vessel wall.. It is from these projections that platelets are formed and the cytoskeleton plays a major role in this. MTs are absolutely required for the extension of PPs but it is the sliding of MTs past each other, driven by the motor protein dynein, and not MT polymerisation, that is the primary driving force of PP extension (Italiano et al. 1999; Patel et al. 2005; Bender et al. 2015). β 1-tubulin is the major isoform of tubulin in MKs and is necessary for PPF (Wang et al. 1986; Schwer et al. 2001). Mutations in β 1-tubulin (*Tubb1*) cause autosomal dominant thrombocytopenia in humans (Kunishima et al. 2009). Although pharmacological disruption of the actin cytoskeleton does not prevent PP extension, actin is involved in the bifurcation of PPs which increases PP tip number (Italiano et al. 1999). More recently the actin cytoskeleton has been implicated in PPF

through the action of WASp in the formation of podosomes, actin rich structures which mediate adhesion and degradation of the extra-cellular matrix (Schachtner et al. 2013) and downstream of PIP₂ present in the MK demarcation membrane system (Schulze et al. 2006).

2.3 Delivery of Cargo into proplatelets

During MK maturation, MKs accumulate platelet-specific granules which are essential to platelet function. During PPF these granules and organelles are distributed into the nascent platelets along bipolar MTs which line the length of the PPs. This movement is via the MT motor kinesin and this drives their capture at the PP tips (Patel et al. 2005; Richardson et al. 2005). In addition to movement along MTs, the sliding of MTs also contributes to the distribution of organelles/granules into platelets. (Richardson et al. 2005).

2.4 Platelet release

The final stage of platelet production occurs in the bloodstream. Anucleate fragments of the PPs, that are larger than individual platelets, bud off into the circulation. Both preplatelets (Thon et al. 2010) and barbell-shaped platelets (Schwartz et al. 2010) have been observed in the circulation and their conversion into platelets is a MT-dependent process. It is thought that preplatelets (discoids of 2-10µm diameter) can reversibly convert into barbell platelets by twisting the MT cytoskeleton around the centre of the preplatelet (Thon et al. 2010). The barbell structures, which have a platelet-sized (2µm) MT coil at each end, can divide into two individual terminal platelets. This fission event is accelerated by the shear forces present in flowing blood (Thon et al. 2010). Mathematical modelling has shown that the cytoskeleton is also responsible for maintaining platelet size (Thon et al. 2012), an important factor in controlling the pro-thrombotic potential of platelets.

3. Cascades

3.1 Rho family small GTPases

The Rho family small GTPases are well characterised regulators of the cytoskeleton, with RhoA, Rac1 and Cdc42 being the best characterised. Historically Cdc42 was thought to drive the formation of filopodia, Rac1 lamellipodia and RhoA stress fibres (Heasman and Ridley 2008). However, it is clear that their roles are more complex than this and this is certainly true in MKs.

Recent data using MK-specific knockouts of RhoA have indicated that it has roles in both MK maturation and platelet formation. During cell division, RhoA recruitment to the cleavage furrow promotes cell division via F-actin and myosin-II contraction. The expression of the Rho-GEFs ECT2 and GEF-H1, which are required for localisation and activation of RhoA at the cleavage furrow, are down-regulated during MK maturation (Gao et al. 2012). The subsequent failure of RhoA-mediated contraction drives polyploidisation via endomitosis. RhoA^{-/-} MKs are larger and have an increased ploidy level when compared to wildtype mice (Suzuki et al. 2013).

The observation that RhoA^{-/-} mice display macrothrombocytopenia (Pleines et al. 2012) indicates an additional role for RhoA in PPF and this may be due to changes in membrane stiffness and deformity (Suzuki et al. 2013). Furthermore, the protein kinase-C isotype epsilon (PKCε) is known to modulate the activity and expression of RhoA and subsequent cytoskeletal rearrangements. Recent observations that knockdown of PKCε in MKs leads to aberrant PPF suggests that localised PKCε-

mediated down-regulation of RhoA activity promotes PPF and platelet release (Gobbi et al. 2013). Overall, the loss of RhoA function leads to aberrant release of large platelets which are rapidly cleared from the circulation. These studies have therefore identified roles for RhoA signalling in both MK maturation and PPF.

Previous studies demonstrated that although platelets from Rac1 deficient mice have defective lamellipodia formation (McCarty et al. 2005; Pleines et al. 2009), Cdc42 seemed to be dispensable for platelet filopodia (Pleines et al. 2010). However, whilst Rac1^{-/-} platelet number and size appeared normal, mice lacking Cdc42 displayed mild thrombocytopenia indicating that it may be required for platelet formation (Pleines et al. 2010). Recent studies with MK-specific knockouts of both Cdc42 and Rac1 have demonstrated that these mice display severe macrothrombocytopenia (Pleines et al. 2013) and identified roles for Rac1/Cdc42 in efficient demarcation membrane formation, synthesis and trafficking of granules and the maintenance of MK structural integrity during maturation (Pleines et al. 2013). Furthermore, they are required for proper MT organisation in PPs, an effect that may well be mediated via the actin-binding protein cofilin (Pleines et al. 2013). These results highlight roles for Rac1/Cdc42 in the coordination of actin and MT cytoskeletons during PPF.

3.2 Rho-GTPase effectors

Many proteins are known to act as downstream effectors of Rho-GTPases to drive actin polymerisation. Recent studies on three proteins have identified roles in regulating MK maturation and platelet formation. The RhoA effector mDia1, a Diaphanous-related formin which regulates both F-actin polymerisation and MT stability (Schönichen and Geyer 2010), is upregulated during MK differentiation in both human and mouse cells (Watkins et al. 2009; Thomas et al. 2011). shRNAi knockdown of mDia1 in CD34+ human MKs resulted in increased PPF, a decrease in F-actin content and an increase in formation of stable MTs (Pan et al. 2014). Furthermore, expression of a constitutively-active mDia1 leads to a decrease in PPF suggesting that activation of mDia1 inhibits PPF (Pan et al. 2014). These data fit well with the observations that RhoA down-regulation is required for PPF and further reinforces the concept that coordination of the actin and MT cytoskeletons is important in these processes.

A second Rho-GTPase effector demonstrated to play a role in platelet formation is Wiskott-Aldrich Syndrome protein (WASp). WASp is classically activated downstream of Cdc42 and mice lacking WASp, or humans with mutations in WASp, display microthrombocytopenia amongst other immune cell abnormalities (Moulding et al. 2013), a phenotype similar to that seen in the Cdc42^{-/-} mice (Pleines et al. 2010). The observation that WASp^{-/-} mice ectopically shed platelets in the bone marrow and additionally lack actin-rich adhesion structures called podosomes (Sabri et al. 2006) was further developed by demonstrations that MK podosomes are important for matrix degradation and protrusion of MKs through ECM basement membranes (Schachtner et al. 2013). Taken together these data show that WASp plays a vital role in ensuring that MKs are releasing platelets into the circulation. Furthermore, the small size of platelets in WAS patients may be due to a WASp-dependent, profilin-mediated effect on MT stability, thus highlighting a new role for WASp as a regulator of MT dynamics (Bender et al. 2014).

Finally, Pak2 (p21-activated kinase2), an effector of Rac1 and Cdc42 GTPases, has been shown to play a role in PPF. MKs from Pak2^{-/-} mice have less β 1-tubulin, decreased F-actin polymerisation, less membrane invaginations and fewer proplatelets than their wild-type counterparts (Kosoff et al.

2015). This study identifies Pak2 as playing a pivotal role in controlling MK actin- and MT-dynamics downstream of Rho-GTPases.

3.3 Actin-binding proteins

There are many actin-binding proteins which function downstream of Rho-GTPase effectors and new insights into several of these proteins in MKs have been identified. MK-specific knockouts of profilin1 display a phenotype similar to that of WASp deficient mice (Bender et al. 2014). Whilst the small size of the profilin1^{-/-} platelets can be explained by effects on MT stability, the thrombocytopenia of profilin1^{-/-} platelets is not due to defective PPF, but rather a combination of i) macrophage-dependent clearance of platelets from the circulation and ii) ectopic production of platelets in the BM. Similar to WASp^{-/-} MKs, Profilin1^{-/-} MKs have reduced podosome formation (Bender et al. 2014) which would reduce the ability of these MKs to protrude across ECM basement membrane and contribute to the thrombocytopenia. Thus the role of profilin in MKs and platelets appears to be downstream of WASp and may explain the mechanism for the microthrombocytopenia observed in WAS patients.

Non-muscle Myosin II (NMII) has been previously shown to have important roles in cytokinesis and cell division (Heissler and Manstein 2013). The failure of the MK to divide following DNA replication is due to a defect in the actin-myosin II contractile ring that is caused by the down regulation of *MYH10* (NMII-B) by the transcription factor RUNX1 (Lordier et al. 2008; Lordier et al. 2012). In contrast, the expression of *MYH9* (NMII-A) increases during maturation and is important for MK organelle distribution, PPF and platelet release (Leon et al. 2007). F-actin organisation and organelle distribution was abnormal in *MYH9*^{-/-} MKs, and NMII-A was required for proper positioning of organelles prior to PPF (Pertuy et al. 2014). Additionally, MK with mutations in *MYH9* displayed normal development (in terms of ploidy and expression of platelet markers), but displayed hyper-contraction (increased stress fibre formation) and a subsequent decrease in PPF (Chen et al. 2013). It is hypothesised that this increased contractility restrains PPF as it could be rescued by inhibition of myosin or Rho Kinase activity. Furthermore, NMII-A is activated under shear stress and plays a role in the shear-induced release of platelets into the circulation (Spinler et al. 2015). Therefore the combined effects of NMII-A and -B regulate several stages of platelet formation and mutations lead to a complex phenotype characterised by macrothrombocytopenia and mild bleeding disorders.

4. Key molecules

Clearly, the regulation of MK development, maturation, PPF and platelet release is complicated, requiring the coordination of both actin- and MT-cytoskeletons. Whilst many of the classical proteins responsible for the regulation of either actin or tubulin have been characterised, it is increasingly clear that many of these proteins play dual roles in this coordination (e.g. WASp, Profilin, mDia1, Rho-GTPases). The recent studies on Pak2 highlight this as its phenotype is complex, with both actin- and MT-dependent effects (Kosoff et al. 2015). Furthermore, understanding how gene expression is regulated during platelet production, specifically the regulation of cytoskeletal-related proteins, will help us to provide a temporal context to their roles. The recent identification of a number of actin-binding proteins regulated by the micro-RNA MiR-142 (Chapnik et al. 2014) is a good example of this and provides foundations for future studies. In addition, recent work reporting proplatelet-independent platelet production during times of acute platelet need (e.g. in thrombocytopenia or inflammation), in which MKs undergo cytoplasmic fragmentation and demonstrate dysregulated MT

organisation (Kowata et al 2014; Nishimura et al. 2015) indicates that there may be alternative cytoskeletal regulatory pathways involved.

5. Pathologies and therapeutic intervention

Defective MK maturation or PPF can result in thrombocytopenia which, if severe, can cause life-threatening bleeding episodes. Understanding the signalling behind the biology of platelet formation is allowing scientists to develop new methods of producing platelets *in vitro* – the holy grail of platelet biology. A major challenge in the study of platelet production is understanding the role that the bone marrow environment plays in the process. As highlighted in Table 1, many studies on the role of individual proteins in platelet production have been carried out using *in vitro* studies where the presence of extracellular matrix proteins, stromal cells and cytokines is limited or non-existent. It is likely that the myriad of signals received from the bone marrow environment modulates platelet formation, as will the shear force from blood flow in the bone marrow sinusoids. Even within an *in vivo* setting, monitoring blood platelet counts at “steady state” in laboratory housed mouse models may mask partial defects in platelet production (for example as has been observed for *Pecam1* KO mice (Dhanjal et al. 2007)). Therefore, studies in both *in vitro* and *in vivo* settings need to be addressed before a protein can be ruled out as playing a role in the process of platelet production. Additionally, whilst studies using knockouts or knockdowns of proteins are useful in understanding the role of a protein, generating mouse models expressing mutant forms of proteins implicated in thrombocytopenia will be important in elucidating the nuances of cytoskeletal regulation of platelet production.

Recently great strides have been made in the production of functional platelets *in vitro* (Nakagawa et al. 2013; Thon et al. 2014; Di Buduo et al. 2015). Further work in this area will facilitate production of large quantities of functional platelets to bypass the limitations of using donated platelets for transfusion. This, coupled with the use of MKs/platelets derived from patient induced-pluripotent stem (iPS) cells (e.g. Ingrungruenglert et al. 2015), will enable the development of both patient-specific therapeutics and models for studying specific diseases.

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Figure Legends and Table**Figure 1 - The stages of platelet production from megakaryocytes.**

1) Immature MKs residing in the osteoblastic niche undergo several rounds of endomitosis to increase their size, ploidy and membrane content whilst migrating to sinusoidal vessels in the vascular niche. 2) Mature MKs degrade the basement membrane using specialised actin structures called podosomes and extend proplatelets by a microtubule-driven process into the bloodstream. 3) Organelles and platelet-specific granules are taken to nascent platelets along bipolar microtubule bundles that line the length of the proplatelet where they become trapped at the tips. 4) The final processing of platelets takes place in the circulation. Preplatelets can reversibly convert to barbell-shaped platelets by twisting the microtubule cytoskeleton before a fission event creates two individual terminal platelets in a process that is potentiated by shear.

Table 1 - Summary of cytoskeletal proteins known to regulate platelet production

Protein	Type of disruption	Species	Platelet number	Platelet size/volume	Other information	Refs
Actin	Pharmacological on MK <i>in vitro</i> - Cytochalasin	Guinea Pig / Mouse	N/A ¹	N/A	Disruption of actin filaments accelerates PP extension, but reduces bifurcation and branching of PP tips.	Tablin et al. 1990; Italiano et al. 1999
Tubulin	Pharmacological on MK <i>in vitro</i> – Nocodazole or Taxol	Guinea Pig / Mouse	N/A	N/A	Inhibition of MTs polymerisation blocks PP extension. Stabilisation of MTs leads to a reduced number of thickened, abnormal PPs.	Tablin et al. 1990; Italiano et al. 1999
	β 1-tubulin knockout	Mouse	Reduced (Approx. 50% of WT)	Normal	Defective PP formation and although platelet size is normal, they lack the discoid shape and have abnormal MT coil.	Schwer et al. 2001
	Patients with β 1-Tubulin mutation (R318W)	Human	Reduced (40-60 x10 ⁹ /litre) ²	Enlarged	R318W β 1-tubulin is expressed but unstable.	Kunishima et al. 2009
RhoA	MK/platelet specific RhoA knockout	Mouse	Reduced (Approx. 50% of WT)	Enlarged (25% larger)	Changes in membrane stiffness and contractility combine to give aberrant release of large platelets which are rapidly cleared.	Pleines et al. 2012; Suzuki et al. 2013
Rac1/Cdc42	MK/platelet specific Cdc42 knockout	Mouse	Reduced (Approx. 50% of WT)	Enlarged (20% larger)	Platelets have shortened lifespan in circulation. Possible role in granule packaging during PPF.	Pleines et al. 2010
	Inducible Rac1 knockout	Mouse	Normal	Normal	No apparent role for Rac1 in platelet production.	McCarty et al. 2005; Pleines et al. 2009
	MK/platelet specific Cdc42/Rac1 double knockout	Mouse	Reduced (Approx. 25% of WT)	Enlarged (25% larger)	Multiple defects in MK maturation and PPF including defective MT organisation in PPs.	Pleines et al. 2013
mDia1	mDia1 knockout	Mouse	Normal	ND ³	No apparent platelet defect in mDia1 knockouts.	Thomas et al. 2011
	shRNAi knockdown of mDia1 in CD34+ primary cells in culture	Human	N/A	N/A	Increased PPF, decreased F-actin and increase in stable MTs. CA ⁴ mDia1 expression gives reduced PPF suggesting mDia1 down-regulation is required for PPF.	Pan et al. 2014

WASp	Patients with WAS ⁵	Human	Reduced	Reduced	Microthrombocytopenia and other immune cell phenotypes.	Moulding et al. 2013
	WASp knockout	Mouse	Reduced	Reduced	Ectopic production of platelets in bone marrow due to failure of podosome formation. Additionally, WASp regulation of profilin-mediated MT stability regulates platelet size.	Sabri et al. 2006; Schachtner et al. 2013; Bender et al. 2014
Pak2	Inducible Pak2 knockout	Mouse	Reduced (Approx. 50% of WT)	Increased (10% larger)	Multiple effects on MK maturation via effects on both actin and MT cytoskeletal regulation.	Kosoff et al. 2015
Non-muscle Myosin II-A	MK/platelet specific NMII-A knockouts	Mouse	Reduced (Approx. 40% of WT)	Enlarged	Abnormal F-actin and organelle distribution in MKs.	Leon et al. 2007; Pertuy et al. 2014
	Patients with <i>MYH9</i> -related disorder	Human	Reduced (10 – 110 x10 ⁹ /litre)	Enlarged	Hyper-contractility of MKs and decreased PPF.	Chen et al. 2013
	Cell lines expressing <i>MYH9</i> -related disorder mutations	Human	N/A ²	N/A	NMII-A activity regulates release of platelets into circulation and determines platelet size.	Spinler et al. 2015
Non-muscle Myosin II-B	shRNAi knockdown of NMII-B in CD34+ primary cells in culture	Human	N/A	N/A	RUNX1-mediated down-regulation of NMII-B leads to endomitosis rather than cell division.	Lordier et al. 2012
Dynein	Pharmacological on MK <i>in vitro</i>	Mouse	N/A	N/A	Dynein is required for MT sliding during PP extension.	Patel et al. 2005; Bender et al. 2015
Kinesin	Kinesin coated beads	Mouse	N/A	N/A	Organelles and granules are transported along MTs by kinesins.	Richardson et al. 2005
PKCε	shRNAi knockdown of PKCε in MK cultures	Mouse	Reduced (in culture platelets)	Enlarged (in culture platelets)	Localised down-regulation of RhoA by PKCε enhances PPF.	Gobbi et al. 2013
ADF/Cofilin	MK/platelet specific ADF/cofilin knockout	Mouse	Reduced (Approx. 5% of WT)	Enlarged	Required for proper formation of the DMS, granule distribution and actin dynamics during MK maturation.	Bender et al. 2010
Profilin	MK/platelet specific Profilin1 knockout	Mouse	Reduced (by Approx. 40%)	Reduced	Ectopic platelet formation in bone marrow possibly due to reduced podosome formation. Effects on platelet size via MT integrity.	Bender et al. 2014

Notes. ¹ N/A = Not applicable. ² Normal human platelet count = 150-400 x10⁹/litre. ³ ND = Not Determined. ⁴ CA = Constitutively active. ⁵ WAS = Wiskott-Aldrich syndrome.

